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Evaluation of an anti-rPA IgG ELISA for measuring the antibody response in mice☆

S.F. Little*, W.M. Webster, S.L.W. Norris, G.P. Andrews

United States Army Medical Research Institute of Infectious Disease, Bacteriology Division, 1425 Porter Street, Fort Detrick, Frederick, MD 21702-5011, USA

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Abstract

A recombinant protective antigen (rPA)-based enzyme-linked immunosorbent assay (ELISA) was developed to measure the serological response of female A/J mice after inoculation with the new rPA-based anthrax vaccine. Several fundamental parameters of the ELISA were evaluated: specificity, precision, accuracy, linearity, and stability. Experimental results suggested that the quantitative anti-rPA IgG ELISA could be used to measure antibody levels in female A/J mice and may be useful as a potency assay to monitor consistency of manufacture of a rPA-based vaccine for planned clinical trials.

Keywords: Enzyme-linked immunosorbent assay; Recombinant vaccine; Anthrax

1. Introduction

Anthrax vaccine, adsorbed Biothrax (AVA, Biothrax; BioPort Corporation, Lansing, MI, USA) is the current vaccine approved for human use against infection with *Bacillus anthracis* spores. The vaccine is prepared from filtered culture supernatants of the V770-NP1-R strain of *B. anthracis* adsorbed to aluminum hydroxide gel (Alhydrogel) and therefore contains various bacterial products adsorbed to the adjuvant. In addition, benzethonium chloride is added as a preservative and formaldehyde is added as a stabilizer. The major immunogen present in the vaccine is protective antigen (PA) [1,2], the central cell-binding component of the anthrax exotoxins. Proteolysis of PA before or after binding to a cell receptor [3–5] results in a cell-bound PA63 fragment that oligomerizes to form heptamers [6]. The heptamers competitively bind lethal factor (LF) and edema factor (EF) to form lethal toxin or edema toxin, respectively [7], and participate in the transport of LF and EF into the cytosol [8]. Current efforts have been directed toward developing a new anthrax vaccine based upon recombinant PA (rPA) adsorbed to Alhydrogel.

Potency assays are one of several tests that are required before the lot release of immunobiologicals. Accepted potency assays are described in various government documents, including the Additional Standards for Bacterial Products in the Code of Federal Regulations, Minimum Requirements, Guidelines for meningococcal and pneumococcal polysaccharide vaccines, and Product License Applications [9]. Animal testing, which includes serological evaluation or protection against challenge, is necessary if the pathogenesis or protective mechanisms against infection are not clearly defined. The current potency assay for evaluating the lot-to-lot consistency of AVA, Biothrax is the relative potency assay. This assay measures the survival time of groups of guinea pigs each inoculated subcutaneously with 1 of 4 dilutions of either the reference vaccine lot or

☆ Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the US Army.

* Corresponding author. Tel.: +1-301-619-4914; fax: +1-301-619-2152

E-mail address: stephen.little@amedd.army.mil (S.F. Little).

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14. ABSTRACT

A recombinant protective antigen (rPA)-based enzyme-linked immunosorbent assay (ELISA) was developed to measure the serological response of female A/J mice after inoculation with the new rPA-based anthrax vaccine. Several fundamental parameters of the ELISA were evaluated: specificity, precision, accuracy, linearity, and stability. Experimental results suggested that the quantitative anti-rPA IgG ELISA could be used to measure antibody levels in female A/J mice and may be useful as a potency assay to monitor consistency of manufacture of a rPA-based vaccine for planned clinical trials.

15. SUBJECT TERMS

Bacillus anthracis, anthrax, enzyme-linked immunosorbent assay, recombinant vaccine, ELISA, laboratory animals, mice

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new lot of AVA, Biothrax and challenged intradermally with Vollum 1B spores of *B. anthracis* 14 days later. Dilutions of the challenge are also tested in an LD_{50} assay as an internal control for the challenge. If the data meet the acceptance criteria of the relative potency model after statistical analysis of the percent survival and time to death, as well as an acceptable challenge dose, the new lot is approved for release. In support of pre-clinical trials with a rPA-based vaccine, we have proposed using a quantitative anti-rPA IgG ELISA to measure the serological response of female A/J mice after vaccination as an alternative to the relative potency assay to evaluate a candidate next-generation anthrax vaccine (Little et al., manuscript in preparation). Data are presented here evaluating various parameters of the quantitative anti-rPA IgG ELISA to support its use.

2. Materials and methods

2.1. rPA

The antigen used for the evaluation procedure was purified rPA obtained commercially from List Biological Laboratories (Campbell, CA, USA) as a lyophilized preparation. Vials were held at 4–6 °C until reconstitution in Milli-Q water to 1 mg/ml. Aliquots were stored at −70 °C. Purified rPA used for preparing antisera and for the affinity purification of ascites was manufactured as a GMP lot by the Biopharmaceutical Production Facility at NCI-FCRC (Frederick, MD, USA).

2.2. Animals

Female A/J and Balb/c mice (Charles River Laboratories, Wilmington, MA, USA) were used when 6–8 weeks old at the start of each experiment. The animals received food and water ad libitum. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

2.3. Anti-anthrax sera

Anti-rPA ascites and anti-AVA Biothrax ascites were produced in female Balb/c mice injected with either rPA mixed with Freund's adjuvant, complete (anti-rPA/FCA ascites), rPA adsorbed to Alhydrogel (anti-rPA/$Al(OH)_3$ ascites), or AVA, Biothrax (anti-AVA ascites) [10]. Mice were injected intraperitoneally (i.p.) with 0.5 ml of 2,6,10,14-tetramethylpentadecane (pristine; Sigma Chemical Co., St Louis, MO, USA) on day 0. Ten days, 17 days, and 38 days later, mice were injected i.p. with either 50 μg of rPA mixed 1:1 with Freund's adjuvant, complete, 50 μg of rPA adsorbed to Alhydrogel at 0.5 mg of aluminum, or 0.5 ml of AVA, Biothrax (lot FAV063) containing 0.6 mg aluminum. On day 42, mice were injected i.p. with $1\times10^6$of Sp2/0-Ag14 myeloma cells. Ascitic fluid was collected from the mice as it was produced. The pooled ascitic fluids were centrifuged at $25{,}000\times g$, passed through 0.2 μm filters, and aliquots were stored at −70 °C. Monoclonal antibody PA I 2D3-3-1 (mAb 2D3) [11,12] was prepared as ascites [11] and stored as above.

For antisera prepared in female A/J mice, rPA was dialyzed against Dulbecco's phosphate buffered saline (DPBS) without calcium or magnesium before vaccine formulation. Adsorption of rPA to Alhydrogel was allowed to occur at 4–6 °C for >2 h before inoculation of animals. Mice ($n=10$) were injected with either 100 μg, 31.6 μg, or 10 μg of rPA adsorbed to 0.1 mg aluminum and antisera were collected at biweekly intervals and were stored at −70 °C (Little et al., manuscript in preparation).

2.4. ELISA

2.4.1. Preparation of reference standard

The standard for the ELISA was prepared from ascites fluids produced in female Balb/c mice injected with rPA mixed with Freund's complete adjuvant (anti-rPA/FCA ascites). The ascites fluids were diluted 1:1 with 10 mM sodium phosphate, 138 mM NaCl, 2.7 mM KCl, pH 7.4 (PBS) before passage over rPA bound to Affi-Gel 15 resin (BioRad Laboratories, Hercules, CA, USA). Bound antibody was eluted with PBS, 50 mM glycine/HCl, 10% ethylene glycol, pH 2.5, neutralized by adding 1 M Tris/HCl, pH 9.0, and dialyzed against PBS. The antibody was then passed over a HiTrap ProteinG column (Pharmacia-Biotech, Piscataway, NJ, USA) and the bound antibody was eluted with 0.1 M glycine/HCl, pH 2.7 and neutralized by adding 1 M Tris/HCl, pH 9.0. The affinity-purified antibody was dialyzed against PBS, filtered through 0.22 μm filters, and aliquots were frozen at −70 °C. Protein concentration, determined by using the BioRad microplate protein assay (BioRad Laboratories), was measured at 1.74 mg/ml. Seven dilutions of the reference standard, three positive controls (high, mid, and low), and a negative control were prepared as two-fold concentrations in PBS containing 0.5% Tween20 and 5% non-fat dry milk and stored at −70 °C. MAb 2D3 was likewise affinity-purified over rPA bound to Affi-Gel 15 resin then chromatographed over a HiTrap Protein G column. Protein was measured at 1.22 mg/ml. Seven dilutions of the affinity purified mAb 2D3, consisting of two-fold concentrations, and two positive controls (high and low)

were prepared in PBS containing 0.5% Tween 20 and 5% non-fat dry milk and stored at −70 °C.

2.4.2. ELISA

The ELISA was designed to measure rPA-specific IgG by using rPA as the solid-phase capture antigen. Each plate contained three triplicate positive controls (high, mid, and low concentrations), one triplicate negative control (normal mouse serum), one triplicate blank, seven, 2-fold dilutions of the reference standard triplicate, and serial dilutions of up to four test samples in triplicate.

Wells of 96-well plates (Immulon IIB, Dynex Technologies, Chantilly, VA, USA) were coated with 100 μl of rPA diluted to 1 μg/ml in PBS overnight at 4 °C. The plates were washed three times with PBS containing 0.1% Tween 20 (PBST) using a Dynex UltrawashPlus microplate washer (Dynex Technologies) before adding 50 μl of reference standards and controls and an equal volume of PBST containing 5% non-fat dry milk (PBSTM) or samples which were serially diluted in PBSTM (100 μl per well). The plates were incubated for 1 h at 37 °C. The plates were washed three times in PBST and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (γ) (Kirkegaard and Perry, Gaithersburg, MD, USA) diluted to 1:1000 in PBSTM was added to the wells and the plates incubated for 1 h at 37 °C. The plates were washed three times with PBST, rotated 180°, and washed again three times before two-component substrate (2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS; Kirkegaard and Perry) was added to the wells. The plates were incubated at 37 °C for 30 min. Stop solution (Kirkegaard and Perry) was added and absorbance readings at 405 nm were obtained using a BioTek 312e microplate reader (BioTek, Winooski, VT, USA). The mean absorbance values, standard deviation, and coefficient of variation (%CV) for each triplicate dilution of all reference standards, controls, and test samples were obtained using the KC4 software program (BioTek Instruments). The IgG concentration of each unknown sample and control was calculated from each corresponding reference standard curve using a 4-parameter logistic regression equation of the KC4 program. Further data analysis was performed using XLfit3 software (IDBS. Inc., Emeryville, CA, USA). The intraclass correlation coefficient (ICC) and 95% confidence interval (95% CI) for the ICC were calculated to assess the degree to which ELISA titers from the linear method (Little et al., manuscript in preparation) and the 4-parameter logistic method were in agreement. ICC values were calculated with SAS Version 8.0 (SAS Institute, Inc., SAS OnlineDoc, Cary, NC, USA) using the equations described by Shrout and Fleiss [13].

2.4.3. Competitive binding study

A competitive binding study was conducted to assess the specificity of the ELISA. Three different ascites preparations used for the study were diluted in PBSTM; anti-AVA ascites (1:40,000), anti-rPA/FCA ascites (1:80,000), and anti-rPA/$Al(OH)_3$ ascites (1:80,000). Ten half-log dilutions of rPA inhibitor were prepared from 200 μg/ml in PBSMT. An equal volume of diluted ascites was added to each rPA concentration and to a tube without rPA. The ascites-rPA solutions were incubated overnight at 4–6 °C on a rotator before analyzing by the ELISA.

3. Results

3.1. Selection of microtiter plates

Three brands of microtiter plates were evaluated to determine which brand demonstrated the best absorbance values in the ELISA. For this comparison, the blank, negative control serum (1:100), and polyclonal positive control sample (1:80,000) were tested in triplicate on each brand of microtiter plate (Table 1). Based upon these results, Immulon 2HB microtiter plates were selected for use in the ELISA.

3.2. Reference standard 4-parameter logistic curve

The relationship between the absorbance values at 405 nm (A_{405}) and the known concentrations of antibody can be plotted by a sigmoid curve defined by a 4-parameter logistic equation [14];

$$y = (A + ((B - A)/(1 + ((X/C)^{D}))))$$

where 'A' is the y-value corresponding to the upper asymptote, 'B' is the y-value corresponding to the lower asymptote, 'C' is the x-value corresponding to the y-midpoint between 'A' and 'B', and 'D' the slope factor of the curve. The reference standard curve consisted of 7, two-fold serial dilutions of affinity-purified mouse anti-rPA IgG from 87 ng/ml (S1, 1:20,000 dilution) to 1.359 ng/ml (S7, 1:1,280,000 dilution). Data from 33 reference standard curves were calculated to be: $A = 3.119 \pm 0.1687$; $B = 0.0843 \pm 0.0347$; $C = 16.91 \pm 2.354$; and $D = -1.430 \pm 0.1483$ for these assays. Fig. 1 depicts the reference standard curve obtained from 33 plates in 11 assays ($r^2 = 0.9998$). For non-immune mouse serum,

Table 1
Absorbance values of blank, negative and positive samples tested in different brands of microtiter plates

Microplate brand and manufacturer	Absorbance values			Difference[a]
	Blank	Negative	Positive	
Reacti-Bind Pierce	0.074	0.365	2.204	1.839
E.I.A. Plus Linbro	0.065	0.166	1.246	1.080
Immulon IIB Dynex	0.071	0.404	2.267	1.863

[a] Difference between positive and negative absorbance values.

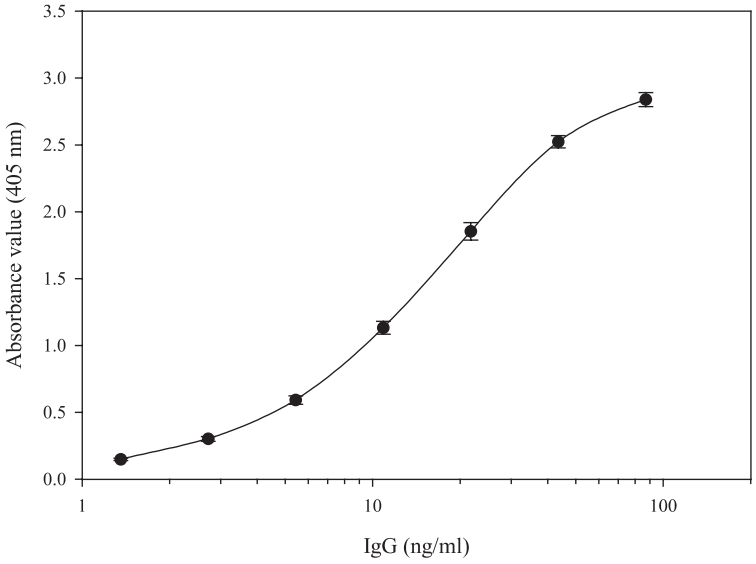

Fig. 1. Four-parameter logistic plot of the average absorbance value ±SD for affinity-purified anti-rPA IgG reference standard prepared from anti-rPA/FCA ascites.

the absorbance value was 0.2406±0.0130 at a 1:100 dilution during the assay.

3.3. Comparison between polyclonal and monoclonal antibody standard

The concentration of three samples, mouse anti-AVA ascites, affinity purified mouse anti-rPA IgG (polyclonal antibody standard), and affinity purified mAb 2D3 (mAb standard) were measured in the ELISA using either affinity purified mouse anti-rPA IgG (1.74 mg/ml IgG) or affinity purified mAb 2D3 (1.22 mg/ml IgG) as the reference standard (Table 2). The ELISA was performed on triplicate dilutions on each of three plates in three separate assays for each test sample for each reference standard ($n=9$). When the reference standard used to quantify the test sample were the same, the anti-rPA IgG concentrations were similar. When anti-rPA was used as the reference standard, the test sample concentrations were greater than when measured using the mAb 2D3 reference standard. However, there was only a significant difference in ELISA titers between the anti-AVA ascites test sample ($P=0.006$). Based upon these results, we selected an affinity purified IgG from polyclonal ascites as a reference standard for the ELISA.

3.4. Specificity

Specificity of the ELISA was evaluated by a competitive binding assay. Fig. 2 shows the inhibition effects of soluble rPA on the binding of anti-AVA ascites, anti-rPA/Al(OH)$_3$ ascites, or anti-rPA/FCA ascites to rPA bound to microtiter plates. Half-maximal binding of anti-AVA ascites and anti-rPA/Al(OH)$_3$ ascites to the plate was measured at 0.55 μg and 0.98 μg of soluble rPA, respectively, whereas 23.23 μg of soluble rPA was required to block 50% binding of anti-rPA/FCA ascites.

Analyte specificity was further assayed by determining the parallelism between the titration curves of the reference standard with three test samples. The relationship between the absorbance value and the corresponding reciprocal of the dilution was made linear by the fully specified logit-log model according to Plikaytis et al. [15]. In determining this relationship the following formula was used,

$$\mathrm{Logit}(A_{405})_{fs} = \log(A_{405}-A_{405min})/(A_{405max}-A_{405})$$

where 'A_{405min}' and 'A_{405max}' are unknown values corresponding to the lower and upper asymptotes, respectively of the 4-parameter logistic-log model [15]. For our calculations, these values were set at 0.1 and 3.1, respectively. The plot between the Logit$(A_{405})_{fs}$ and the respective log of the reciprocal of the dilution was a straight line. The slopes measured for the curves from five separate assays of three plates per assay were: reference standard, −1.4445; anti-AVA ascites, −1.3060; anti-rPA/Al(OH)$_3$ ascites, −1.5024; and anti-rPA/FCA ascites, −1.4879. The differences between the slopes of the reference standard and AVA ascites, rPA/Al(OH)$_3$ ascites, and rPA/FCA ascites were 0.1385, 0.0579, and 0.0434, respectively.

3.5. Precision

The intra-assay and inter-assay precision was evaluated for the three positive control samples at three different concentrations. Within assay precision for three plates per assay and inter-assay precision from

Table 2
Comparison between affinity-purified polyclonal anti-rPA IgG and affinity-purified mAb 2D3 IgG reference standards in determining the concentration of selected test samples

Reference standard	Test sample concentration (μg IgG per ml)		
	Anti-AVA	Anti-rPA[a]	mAb 2D3[b]
Anti-rPA 1.74 mg/ml IgG	625.06	1806.1	1410.4
	440.55	1330.3	1184.3
	455.29	1442.0	1060.1
	592.85	1762.7	1340.1
	622.98	1855.5	1488.7
	646.28	2017.9	1552.6
	621.66	2059.2	1823.7
	640.16	1898.8	1651.1
	575.94	2230.0	1623.0
Average	580.1	1822.5	1459.3
%CV	13.5%	15.7%	16.4%
mAb 2D3 1.22 mg/ml IgG	346.16	1171.3	984.14
	341.34	1354.1	1038.3
	382.46	1152.9	1014.4
	471.54	1455.4	1322.1
	499.02	1759.2	1350.0
	472.41	1706.7	1342.2
	544.31	2150.5	1599.0
	559.50	1747.3	1390.0
	524.25	1506.7	1187.2
Average	460.1	1556.0	1247.5
%CV	18.2%	20.5%	16.5%
Significance[c]	$P=0.006$	$P=0.081$	$P=0.061$

[a] Affinity purified anti-rPA IgG reference standard.
[b] Affinity purified mAb 2D3 IgG reference standard.
[c] Significance between each test sample as measured for each reference standard (*t*-test).

30 plates run in ten assays is shown in Table 3. Intra-assay %CVs for high-, mid-, and low-concentration, positive control samples ranged from 0.1% to 12%. Inter-assay precision for the ten assays ranged from 13.5% for the high positive control, 11.0% for the mid positive control, and 8.0% for the low positive control. In addition, the average %CVs of the triplicate determinations for the seven standards (S1–S7) from 33 reference standard curves in 11 assays were (S1) 1.8 ± 1.82, (S2) 1.8 ± 2.01, (S3) 3.5 ± 3.98, (S4) 4.2 ± 5.02, (S5) 5.4 ± 6.57, (S6) 6.0 ± 7.66, and (S7) 5.9 ± 8.34%.

3.6. Accuracy and linearity

Because there is no accepted reference standard mouse anti-rPA IgG, anti-rPA IgG was affinity purified from a pooled mouse anti-rPA/FCA ascites and used as a working reference standard. The ascites was prepared in female Balb/c mice inoculated with rPA mixed with Freund's complete adjuvant as described in the methods section. Normal mouse serum was spiked with 1:5, 1:50, and 1:500 dilutions of the affinity-purified mouse anti-rPA IgG. Two different analysts ran the samples in triplicate on each of three plates under the same conditions on the same day. The observed concentrations were in close agreement with the nominal concentrations. For the six plates, the percent recovery was 92.3% (1:5 dilution), 112.1% (1:50 dilution), and 89.8% (1:500 dilution).

3.7. Stability

To evaluate the stability of rPA as a capture antigen on microtiter plates, the ELISA was conducted with plates that had been coated either 3 days or 10 days in advance and held at 4–6 °C and compared with plates that had been coated 18–20 h ahead of time under standard assay conditions. Samples, which consisted of the high, mid, and low positive controls, were run in triplicate on each of three plates in duplicate assays on the same day. Results showed that there was no discernible difference in concentrations between similar samples for plates prepared 3 days and 10 days beforehand (Table 4).

Further analysis of stability of rPA was evaluated under freeze/thaw cycle conditions. Two aliquots of rPA were treated by freezing/thawing at −70 °C to room temperature for three times and were used to prepare three microtiter plates each. For the analysis, the three positive controls were run on each plate. Results indicated that there was no discernible difference in the mean absorbance values for the same antibody concentrations, suggesting that rPA is stable at these temperature and buffer conditions (Table 5).

3.8. Agreement between ELISA titers determined by 4-parameter and linear analysis

A comparison was made between week 5 ELISA titers that had been determined by linear regression analysis (Little et al., manuscript in preparation) with titers determined by 4-parameter logistic regression analysis. Sera were tested from ten female A/J mice per group that had been injected with 100 μg, 31.6 μg, or 10 μg of rPA adsorbed to Alhydrogel and bled for serology. The titers were calculated from the same two dilutions from each sample titration. The average coefficient of determination (r^2) for the reference standard from ten plates plotted by linear regression analysis was $r^2=0.9878$ and by 4-parameter logistic regression analysis it was $r^2=0.9985$. There was a high degree of agreement between the two methods. The intraclass correlation coefficient was 0.9916 (95% CI 0.9823–0.9960).

4. Discussion

We recently proposed using a quantitative anti-PA IgG ELISA that was developed to measure the antibody

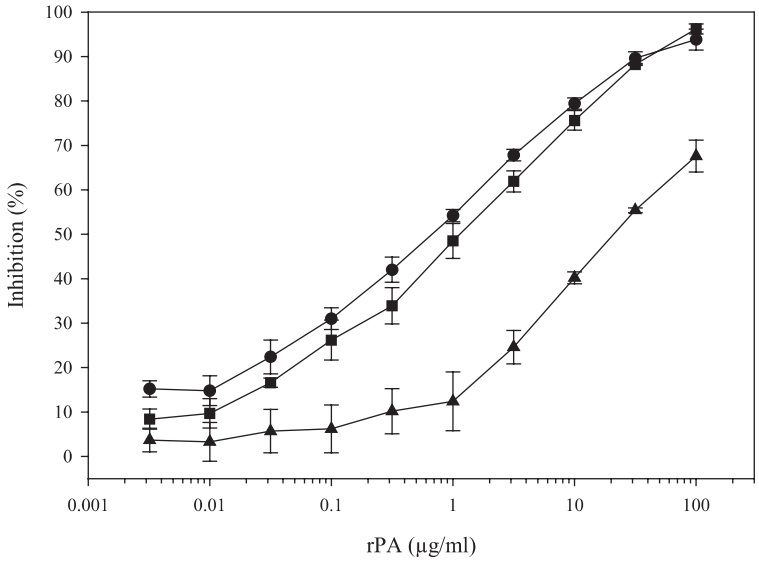

Fig. 2. Inhibition of soluble rPA on binding of anthrax antisera to rPA bound to wells of microtiter plates. Anti-AVA ascites (●), anti-rPA/$Al(OH)_3$ ascites (■), and anti-rPA/FCA ascites (▲).

response of female A/J mice inoculated with rPA adsorbed to Alhydrogel as a potency assay for the rPA anthrax vaccine candidate in support of clinical trials (Little et al., manuscript in preparation). The ELISA that was developed was based upon previous studies, including a GLP clinical trial [16] and an animal experiment [17]. In these ELISAs, the plot between the dilutions of the standard reference serum and the respective absorbance values was a straight line. The first sample absorbance value that could be read off the linear portion of the standard curve was used to determine the sample concentration by linear regression analysis. One of the acceptance criteria that were established for the ELISA was that the linear standard curve had to have an r^2 value of >0.9700. In this study, we substituted a 4-parameter logistic model for the linear regression model that was originally used to develop the proposed mouse potency assay (Little et al., manuscript in preparation) and found that the 4-parameter logistic model gave a better fit for the reference standard data points, which ranged from 34.8 ng/ml (S1) to 4.35 ng/ml (S7). This was shown by higher r^2 values for the reference standard curve when the absorbance values were fit to a 4-parameter logistic curve. When the 4-parameter model was used to re-calculate the concentration of anti-PA IgG in the sample sera previously determined by linear regression analysis, good agreement was measured with titers from the previous study (ICC = 0.9916).

Table 3
Precision determination for the quantitative mouse anti-rPA IgG ELISA using three different concentrations of the positive control sera[a]

Assay	Positive control serum dilution and concentration					
	High		Mid		Low	
	1:80,000 21.75 ng/ml		1:130,000 13.38 ng/ml		1:220,000 7.91 ng/ml	
	Concentration (ng/ml)	%CV	Concentration (ng/ml)	%CV	Concentration (ng/ml)	%CV
1	19.95 ± 0.162	0.8	12.61 ± 0.110	0.9	7.88 ± 0.300	3.8
2	21.92 ± 0.168	0.8	13.45 ± 0.073	0.5	8.25 ± 0.010	0.1
3	20.69 ± 0.514	2.5	12.71 ± 0.237	1.9	7.90 ± 0.112	1.4
4	20.98 ± 0.316	1.5	12.80 ± 1.141	1.1	7.81 ± 0.247	3.2
5	21.38 ± 0.082	0.4	13.01 ± 0.180	1.4	8.01 ± 0.102	1.3
6	28.21 ± 3.373	12.0	17.42 ± 1.220	7.0	9.64 ± 0.604	6.3
7	26.10 ± 0.557	2.1	14.14 ± 0.548	3.9	8.33 ± 0.096	1.2
8	19.07 ± 1.528	8.0	12.90 ± 0.427	3.3	7.12 ± 0.341	4.8
9	20.77 ± 0.126	0.6	12.62 ± 0.226	1.8	8.16 ± 0.263	3.2
10	20.34 ± 0.271	1.3	12.68 ± 0.184	1.5	8.07 ± 0.166	2.1
Average concentration (ng/ml)	21.94 ± 2.971	13.5	13.43 ± 1.479	11.0	8.12 ± 0.651	8.0

[a] Three plates were run for each assay.

Table 4
Stability of the ELISA for mouse anti-rPA IgG positive controls using plates coated with rPA 3 days and 10 days earlier

Sample	18–20 hour plates			3-day-old plates			10-day-old plates		
	IgG (ng/ml)	SD	%CV	IgG (ng/ml)	SD	%CV	IgG (ng/ml)	SD	%CV
High	21.3	0.757	3.6	22.4	1.038	4.6			
Mid	13.1	0.435	3.3	13.5	0.462	3.4			
Low	8.1	0.206	2.5	8.1	0.204	2.5			
High	22.6	3.985	17.6				21.1	2.264	10.7
Mid	13.5	0.809	6.0				13.6	0.981	7.2
Low	7.7	0.697	9.0				8.0	0.390	4.9

For the evaluation of the ELISA, we prepared reference standards between 87 ng/ml (S1) and 1.359 ng/ml (S7) to optimize the 4-parameter logistic curve. Evidence that supported using the ELISA to measure the antibody levels to rPA was shown by the small differences between the slopes (parallelism) of the curves from the reference standard and three test samples. Other experimental results from this study suggested that the ELISA had excellent specificity, precision, accuracy, and linearity. Low %CV values were measured from well-to-well, plate-to-plate, and assay-to-assay data for the three positive controls. Normal serum that was spiked with the reference standard and tested in the ELISA resulted in concentrations that were in close agreement with the target concentrations.

The difference between binding of anti-rPA/FCA ascites and anti-rPA/$Al(OH)_3$ ascites and anti-AVA ascites to rPA bound to wells of microtiter plates in the presence of soluble rPA may have been due to adjuvant effects on epitope specificity or antibody affinity. Both anti-rPA/$Al(OH)_3$ ascites and anti-AVA ascites, which had similar 50% inhibition values, contained rPA adsorbed to Alhydrogel (aluminum hydroxide) while anti-rPA/FCA included a water-in-oil emulsion adjuvant. It took about 20- to 40-fold less soluble rPA to inhibit 50% binding of anti-rPA/$Al(OH)_3$ ascites and anti-AVA ascites to rPA bound to wells of microtiter plates than it did for anti-rPA/FCA ascites. Freund's complete adjuvant has been reported to induce antibodies to denatured epitopes [18]. Likewise, protein bound to the surface of microtiter plates often is denatured or undergoes a conformational change [19–21]. It could be postulated then, that the anti-rPA/FCA ascites has a higher affinity to denatured epitopes as presented on the ELISA plate than for native epitopes on soluble rPA. Additionally, we noted in our laboratory that antisera produced with Alhydrogel generally have higher neutralizing titers against lethal toxin cytotoxicity than antisera produced in the presence of FCA (personal observations).

Antiserum is normally isolated from the blood of animals previously inoculated with the selected reagent combined with an appropriate adjuvant, when necessary. A drawback in producing murine antiserum is that a large amount of antigen is necessary to inoculate the required number of animals needed to acquire the desired amount of blood from which to separate serum. We used the method of Lacy and Voss [10] to produce polyclonal ascites fluids and were able to obtain a large volume of antibody-containing ascites fluid from a relatively small number of animals and quantity of antigen. We routinely collected between 170–200 ml of high titer ascites fluid from 20 mice. An argument supporting the use of monoclonal antibodies as the reference standard can be made in that the material would be obtained from hybridoma cell lines which might reduce the variability that might occur during the preparation of polyclonal ascites fluid. However, the ELISA titers measured when using a monoclonal antibody as the standard may yield lower titers compared to when a polyclonal antibody is used.

In summary, experimental results show that data from a quantitative anti-rPA IgG ELISA calculated by 4-parameter logistic analysis had good specificity, precision, accuracy, and linearity, was in good agreement with previously published findings, and support using this in vitro serological assay to measure antibody levels in mice.

Table 5
Stability of the ELISA for mouse anti-rPA IgG positive controls using plates coated with rPA after three F/T cycles

Sample	18–20 hour plates			3×F/T sample 1			3×F/T sample 2		
	A_{405}	SD	%CV	A_{405}	SD	%CV	A_{405}	SD	%CV
High	1.8899	0.20783	11.0	1.8329	0.09689	5.3	1.9910	0.09103	4.6
Mid	1.3340	0.19247	14.4	1.3310	0.14048	10.6	1.4634	0.12255	8.4
Low	0.8197	0.12271	15.0	0.8529	0.11944	14.0	0.9133	0.13251	14.5

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References

[1] Ivins BE, Welkos SL. Recent advances in the development of an improved, human anthrax vaccine. Eur J Epidemiol 1988;4:12–9.
[2] Ivins BE, Welkos SL, Knudson GB, Little SF. Immunization against anthrax with aromatic compound-dependent (Aro^-) mutants of *Bacillus anthracis* and with recombinant strains of *Bacillus subtilis* that produce anthrax protective antigen. Infect Immun 1990;58:303–8.
[3] Bradley KA, Mogridge J, Mourez M, Collier RJ, Young JAT. Identification of the cellular receptor for anthrax toxin. Nature 2001;414:225–9.
[4] Klimpel KR, Molloy SS, Thomas G, Leppla SH. Anthrax toxin protective antigen is activated by a cell surface protease with the sequence specificity and catalytic properties of furin. Proc Natl Acad Sci U S A 1992;89:10277–81.
[5] Ezzell JW Jr, Abshire TG. Serum protease cleavage of *Bacillus anthracis* protective antigen. J Gen Microbiol 1992;138:543–9.
[6] Milne JC, Furlong D, Hanna PC, Wall JS, Collier RJ. Anthrax protective antigen forms oligomers during intoxication of mammalian cells. J Biol Chem 1994;269:20607–12.
[7] Leppla SH. The anthrax toxin complex. In: Alouf JE and Freer JH (Eds). Sourcebook of bacterial protein toxins. New York: Academic Press, 1991;277–302.
[8] Little SF, Ivins BE. Molecular pathogenesis of *Bacillus anthracis* infection. Microbes Infect 1999;2:131–9.
[9] Habig WH. Potency testing of bacterial vaccines for human use. Vet Microbiol 1993;37:343–51.
[10] Lacy MJ, Voss EW Jr. A modified method to induce immune polyclonal ascites fluid in BALB/c mice using Sp2/0-Ag14 cells. J Immunol Methods 1986;87:169–77.
[11] Little SF, Leppla SH, Cora E. Production and characterization of monoclonal antibodies to the protective antigen component of *Bacillus anthracis* toxin. Infect Immun 1988;56:1807–13.
[12] Little SF, Novak JM, Lowe JR, Leppla SH, Singh Y, Klimpel KR, et al. Characterization of lethal factor binding and cell receptor binding domains of protective antigen of *Bacillus anthracis* using monoclonal antibodies. Microbiology 1996;142:707–15.
[13] Shrout P, Fleiss J. Intraclass correlations: uses in assessing rater reliability. Psychol Bull 1979;86:420–8.
[14] O'Connell M, Belanger B, Haaland P. Calibration and assay development using the four-parameter logistic model. Chemom Intell Lab Sys 1993;20:97–114.
[15] Plikaytis BD, Holder PF, Pais LB, Maslanka SE, Gheesling LL, Carlone GM. Determination of parallelism and nonparallelism in bioassay dilution curves. J Clin Microbiol 1994;32:2441–7.
[16] Pittman PR, Kim-Ahn G, Pifat DY, Coonan K, Gibbs P, Little S, et al. Anthrax vaccine: immunogenicity and safety of a dose-reduction, route-change comparison study in humans. Vaccine 2002;20:1412–20.
[17] Pitt MLM, Little SF, Ivins BE, Fellows P, Barth J, Hewetson JGP, et al. In vitro correlate of immunty in a rabbit model of inhalational anthrax. Vaccine 2001;19:4768–73.
[18] Jennings VM. Review of selected adjuvants used in antibody production. ILAR J 1995;37:119–24.
[19] Hollander Z, Katchalski-Katzir E. Use of monoclonal antibodies to detect conformational alterations in lactate dehydrogenase isoenzyme 5 on heat denaturation and on adsorption to polystyrene plates. Mol Immunol 1986;23:927–33.
[20] Jitsukawa T, Nakajima S, Sugawara I, Watanabe H. Increased coating efficiency of antigens and preservation of original antigenic structure after coating in ELISA. J Immunol Methods 1989;116:251–7.
[21] Vaidya HC, Dietzler DN, Ladenson JH. Inadequacy of traditional ELISA for screening hybridoma subernatants for murine monoclonal antibodies. Hybridoma 1985;4:271–6.